Protocol: Sandwich ELISA (Colorimetric) – Direct Detection

Related Links

ELISA Protocols, Troubleshooting, & Scientific Resources

ELISA Metrics Infographic

View all protocols

Capture and detection antibodies should be matched for an ELISA. They should not bind the same epitope or recognize epitopes in close proximity.

MATERIALS

- **1X Phosphate Buffered Saline (PBS):** Dissolve 1.44 g Na_2HPO_4 (10 mM), 0.24 g KH_2PO_4 (1.8 mM), 8 g NaCl (137 mM), and 0.2 g KCl (2.7 mM) in 800mL of diH_20. Adjust the pH to 7.4 with 0.1N HCl, and add diH_20 up to 1L.
- **Coating Buffer:** Dissolve anhydrous 5.3 g Na_2CO_3 (50mM) and anhydrous 4.2 g $NaHCO_3$, (50mM) in diH_20. Adjust pH to 9.6 and add diH_20 up to 1L.
- **Reagent Diluent:** Dissolve BSA in Phosphate Buffered Saline (1X PBS) to 1% concentration (w/v).
- **Wash buffer:** 1X PBS containing 0.05% v/v Tween-20
- **Capture Antibody Solution:** Add the primary capture antibody to the Coating Buffer. If a working antibody concentration is not available, use the below table for recommended starting concentrations. The clonality of the antibody will impact the suitable concentration range.

	Monoclonal Capture/ Polyclonal Detection	Monoclonal Capture/ Monoclonal Detection	Polyclonal Capture/ Polyclonal Detection
Capture Antibody	1-8 µg/mL	0.5-4 µg/mL	0.2-0.8 µg/mL
Detection Antibody	50-400 ng/mL	0.25-2 µg/ml	50-400 ng/mL

- **Detection Antibody Solution:** Add the enzyme conjugated detection antibody to the Reagent Diluent. Common enzyme conjugates for ELISAs include Horse Radish Peroxidase (HRP) and Alkaline Phosphatase (AP). If a working antibody concentration is not available, use the above table for recommended starting concentrations. The clonality of the antibody will impact the suitable concentration range.
- **Substrates and Stop Solutions:** Follow datasheets for preparation of Substrate Solutions.

Conjugates	Substrates	Stop Solution	Absorbance Settings
HRP	TMB (3,3′,5,5′-Tetramethylbenzidine)	2M H_2SO4	450nm
AP	PNPP (Para-Nitro Phenyl Phosphate)	1M NaOH	405nm

SAMPLE PREPARATION

Dilute the experimental samples (serum, plasma, lysates or other biofluids) and perform a serial dilution of the standard (purified form of the analyte under consideration) in Reagent Diluent. If available, use the literature to determine the optimal concentration and linear working range. Samples should be prepared in duplicate, or preferably in triplicate.

METHODS

1. Use an ELISA compatible PVC clear microtiter plate (96-well) and determine the number of wells needed for the assay.

2. Dispense 100 µl of Capture Antibody Solution into the wells. Apply sealing tape to the top of the plate to prevent evaporation. Incubate the plate overnight at 4°C. After incubation remove the

out the solution. Wash the plate 2 more times and pat the inverted plate on a paper towel to dry.

4. Block plates by dispensing 300 µl of Reagent Diluent into each well and incubate the plate for 60 minutes at room temperature. After incubation, remove the tape. Invert the plate and flick out the solution.
5. Repeat step 3.
6. Add 100 µl of diluted samples and standards to the appropriate wells and cover the plate.
7. Incubate the plate for 2 hours at room temperature. After incubation, uncover the plate and aspirate each well.
8. Repeat step 3.
9. Dispense 100 µl of Detection Antibody Solution to each well. Incubate for 2 hours at room temperature and cover the plate. After incubation, uncover the plate. Invert the plate and flick out the solution.
10. Repeat step 3.
11. Dispense 100 µl of the Substrate Solution into each well. Cover the plate and incubate for 20 to 30 minutes at room temperature. Avoid placing the plate in direct sunlight.

    Tip: If no color develops within 30 minutes, incubation time can be extended until an appropriate color change/signal is acquired. TMB substrate reacts with HRP to generate a blue colored product which changes to yellow upon the addition of Stop Solution, whereas the PNPP substrate reacts with ALP to generate a yellow colored product.

12. After color development, remove the cover and dispense 50 µl of Stop Solution into each well to stop the enzymatic reaction.
13. Immediately measure the absorbance of each well using a spectrophotometer/plate reader with the appropriate absorbance setting.

    Tip: The presence of air bubbles in wells may impact the results and it is advisable to spin the plate in a centrifuge.